

Tetraspanin-3 regulates protective immunity against *Eimeria tenella* infection following immunization with dendritic cell-derived exosomes

Emilio del Cacho ^{a,*}, Margarita Gallego ^a, Hyun S. Lillehoj ^b, Joaquin Quilez ^a, Erik P. Lillehoj ^c, Caridad Sánchez-Acedo ^a

^a Department of Animal Pathology, Faculty of Veterinary Sciences, University of Zaragoza, Zaragoza, Spain

^b Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705, USA

^c Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD 21201, USA

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ABSTRACT

The effects of immunization with dendritic cell (DC) exosomes, which had been incubated with a tetraspanin-3 (Tspan-3) blocking antibody (Ab) or with an isotype-matched non-immune IgG, were studied using an experimental model of *Eimeria tenella* avian coccidiosis. Purified exosomes from cecal tonsil and splenic DCs expressed Tspan-3 protein. Chickens injected with exosomes incubated with the control IgG and derived from cecal tonsil DCs preloaded *in vitro* with *E. tenella* Ag had Ag-immunostaining cells in the ceca, but not the spleen. Conversely, Ag-containing cells were found only in the spleen, but not the ceca, of chickens given IgG treated splenic DC exosomes. Interestingly, chickens that received exosomes incubated with Tspan-3 Ab had Ag-containing cells observed in both lymphoid organs following administration of exosomes from either DC population. After injection of exosomes non-incubated with Tspan-3 Ab, greater numbers of cells secreting interleukin-2 (IL-2), IL-16, interferon- γ , and *E. tenella*-reactive Abs were observed in the cecal tonsils of chickens immunized with cecal DC exosomes compared with the spleen. By contrast, more cytokine- and Ab-producing cells were present in the spleen of chickens given splenic DC exosomes compared with the ceca. Incubation with Tspan-3 Ab gave similar numbers of cytokine- and Ab-producing cells in the cecal tonsils and spleen regardless of the source of exosomes. Immunization with *E. tenella* Ag-loaded cecal tonsil DC exosomes increased *in vivo* resistance against subsequent *E. tenella* infection. Increased protection against infection following cecal DC exosome immunization was partially blocked by incubation of exosomes with Tspan-3 Ab. These results suggest that Tspan-3 is involved in the tissue distribution, as well as cytokine and Ab production, following DC exosome administration, and that Tspan-3 contributes to *in vivo* protection against experimental *E. tenella* challenge infection following exosomal immunization.

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1. Introduction

Exosomes are 30–100 nm diameter extracellular particles originating from the fusion of multivesicular cytoplasmic bodies with the plasma membrane prior to their secretion [1–4]. The role of exosomes in Ag presentation and immune modulation has been demonstrated during *Salmonella* [5], *Mycobacterium* [6], *Toxoplasma* [7], and *Leishmania* [8] infections. Exosomes purified from Ag-presenting cells activate T cells [9], preferentially eliciting

polarized Th1 immune responses [7]. On the basis of these results, Ag-loaded exosomes have been considered as vaccine candidates against a variety of infectious diseases, including *Eimeria tenella* infections. It is well known that Th1 cell-mediated immunity is the main component of protective immunity against *Eimeria* parasites [10–12].

Following their release into the extracellular milieu, exosomes from Ag-presenting cells travel to and accumulate within lymphoid organs for dissemination and targeted delivery of their cargo Ags [13,14]. While the molecular and cellular mechanisms of selective exosome capture by lymphocytes are poorly understood, exosome surface proteins appear to play a key role in this process. Proteins present on the exosome surface include tetraspanins (Tspans) [15,16]. Tspans are members of a family of membrane-tethered proteins characterized by four transmembrane domains,

* Corresponding author at: Parasitología y Enfermedades Parasitarias, Facultad de Veterinaria, Miguel Servet 177, 50013-Zaragoza, Spain. Tel.: +34 976 7615 56; fax: +34 976 76 16 12.

E-mail address: edelcach@unizar.es (E. del Cacho).

intracellular NH₂— and COOH-termini, and two extracellular loops [17]. Tspans are widely distributed in mammalian cells and homologous, conserved proteins are found as distant as the trematodes [18,19].

Recent studies suggest that Tspans play a role in target cell selection by exosomes [14,20]. More specifically, because Tspan-3 is expressed by murine DCs and controls T cell priming [21], we hypothesized that Ab-mediated blockade of Tspan-3 on avian DC exosomes would inhibit their ability to stimulate Ag-specific immune responses by impairing exosome cell-specificity. To test this hypothesis, cecal and splenic DCs were loaded *in vitro* with Ag from *Eimeria* protozoa, and their exosomes were isolated, incubated with Tspan-3 Ab or non-immune IgG, and assessed for Tspan-3 expression as well as their tissue distribution and ability to regulate *in vitro* and *in vivo* parameters of protection against parasite infection.

2. Materials and methods

2.1. Animals

Male White Leghorn chickens were hatched and reared under *Eimeria*-free conditions with *ad libitum* access to feed and water. All experiments were performed in accordance with guidelines approved by the University of Zaragoza Institutional Animal Care and Use Committee.

2.2. Parasite

An *E. tenella* strain originally obtained from Merck, Sharp, and Dome (Spain) was used. Oocysts were propagated, isolated, and sporulated using standard procedures [22]. Chickens were infected with sporulated oocysts by oral inoculation into the crop [23].

2.3. DC isolation

Cecal tonsils and spleens from *E. tenella*-infected chickens were homogenized for 1 h at 37 °C in GKN buffer [24]. CD45⁺ DCs were isolated by fluorescence-activated cell sorting (FACS) and streptavidin-conjugated magnetic beads as described [25].

2.4. *E. tenella* Ag purification and DC loading

Freshly excysted sporozoites were purified from oocyst and sporocyst debris, and sonicated on ice as described [25]. The sonicate was centrifuged at 300 × g for 5 min and the *E. tenella* Ag was purified as described [26]. Protein concentrations were determined according to the procedure by Lowry et al. [27] using BSA as standard, and stored at –20 °C. Purified DCs (5.0×10^5) were pulsed with 50 µg of sporozoite Ag at 39 °C for 1 d.

2.5. Purification of Ag-pulsed DC exosomes

E. tenella Ag-pulsed DCs were exposed to a non-lethal heat shock at 43.5 °C for 30 min followed by a recovery period at 41 °C (the basal temperature of the chicken) for 2 h [28]. Cell debris was removed from culture supernatants by centrifugation at 300 × g for 10 min at 4 °C. Culture supernatants were filtered and exosomes were isolated as described [26].

2.6. Immunoelectron microscopy

Purified DC exosomes were applied to collodion-coated grids and fixed in 0.05% glutaraldehyde. The grids were incubated for 18 h with Tspan-3 Ab (1:10 dilution), or a species-matched, non-immune IgG negative control, followed by incubation for 2 h with

5 nm gold-conjugated goat anti-rabbit IgG secondary Ab. Exosomes were stained with phosphotungstic acid [29].

2.7. Exosome immunization

One-day-old chickens ($n = 144$) were evenly distributed over 6 groups of 24 chickens based on their body weight. Assignment of treatment to each group was at random based on a computer generated list, using Microsoft Excel 2007. At 7 d of age, chickens in groups 1–4 were injected intramuscularly with 100 µl of purified exosomes from Ag-pulsed cecal tonsil (groups 1 and 2) or spleen (groups 3 and 4) DCs (10 µg/bird). Prior to immunization, exosomes were incubated for 90 min at room temperature with rabbit Tspan-3 Ab (1:100 dilution) (Sigma, St. Louis, MO) (groups 1 and 3) or with non-immune rabbit IgG (groups 2 and 4). Chickens in groups 5 and 6 were injected intramuscularly with 100 µl of sterile PBS as unimmunized controls. Chickens in group 5 served as infection control in order to assess the effect of infection on unimmunized birds (unimmunized and infected control). Chickens in group 6 (unimmunized and uninfected) served as control for zootechnical parameters. At 15 d of age, 6 chickens from each group were euthanatized and cecal tonsils and spleens were removed for immunofluorescence localization of sporozoite Ag, flow cytometric quantification of sporozoite Ag-containing cells, and enzyme-linked immunosorbent spot (ELISPOT) quantification of Ag-specific cytokine- and Ab-secreting cells.

2.8. Immunofluorescence microscopy

Cecal tonsils and spleen were frozen in liquid nitrogen and cryostat sections were blocked with normal horse serum for 10 min followed by incubation with *E. tenella* antiserum (1:100 dilution) for 18 h at 4 °C and Alexa Fluor 350-conjugated goat anti-rabbit IgG secondary Ab for 30 min (Molecular Probes, Eugene, OR). Nuclei were stained with anti-DNA Ab (Santa Cruz Biotechnology, CA) followed by incubation with fluorescein-conjugated goat anti-mouse IgG secondary Ab for 30 min (Vector). Cecal tonsil sections from uninfected chickens and sections from *E. tenella*-infected chickens were stained with normal rabbit serum to use as negative controls.

2.9. Fluorescence-activated cell sorting

Mononuclear cells were isolated from cecal tonsils and spleens by density gradient centrifugation as described [25]. The cells (5.0×10^6) were sequentially incubated with normal rabbit serum (Vector) for 10 min, 0.2 ml of *E. tenella* rabbit Ab or species-matched control Ab (1:100 dilution) for 90 min, and fluorescein-conjugated goat anti-rabbit IgG secondary Ab (Vector) (1:100 dilution) for 30 min. FACS was performed according to the procedure described in [25] and [30].

2.10. ELISPOT assays for cytokine- and Ab-secreting cells

Cecal tonsils and spleens were harvested at 8 d post-immunization. Single-cell suspensions were obtained by filtering through a 70-µm-pore-size cell strainer (BD Falcon) and were purified by Percoll density gradient centrifugation as described above. For quantification of cytokine-secreting cells, 1.0×10^5 cells were cultured and ELISPOT assays were performed as described [25]. For quantification of Ab-secreting cells ELISPOT assays were performed as described [30]. Negative controls contained cells incubated with medium alone in the absence of *Eimeria* Ag.

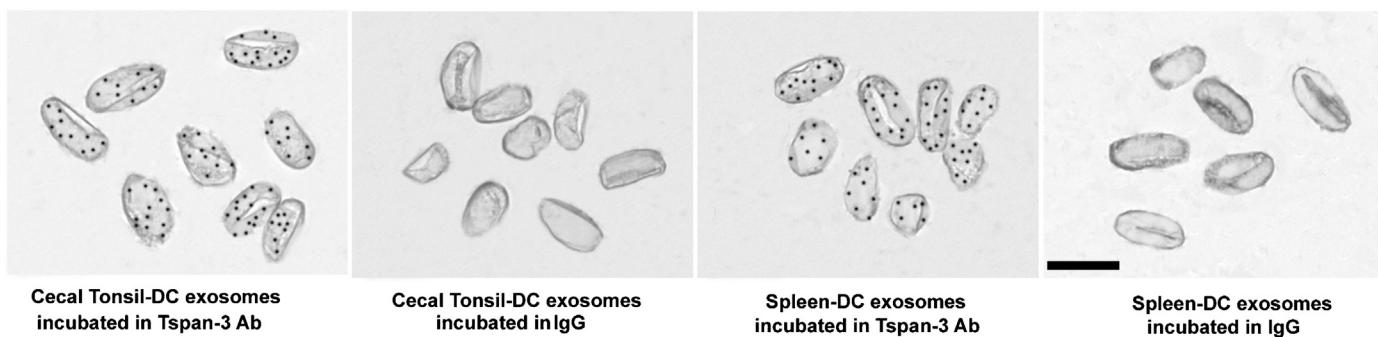


Fig. 1. Tspan-3 immunoelectron microscopy of exosomes derived from cecal tonsil- or splenic DCs loaded with Ag. Micrographs are whole mount of exosomes showing vesicles 80–100 nm in diameter, devoid of cellular debris, and immunogold-stained with rabbit anti Tspan-3 Ab, or non-immune rabbit IgG (control). Note the gold particles on the exosomes from cecal tonsil and splenic DCs show an identical pattern of distribution. Bar = 100 nm.

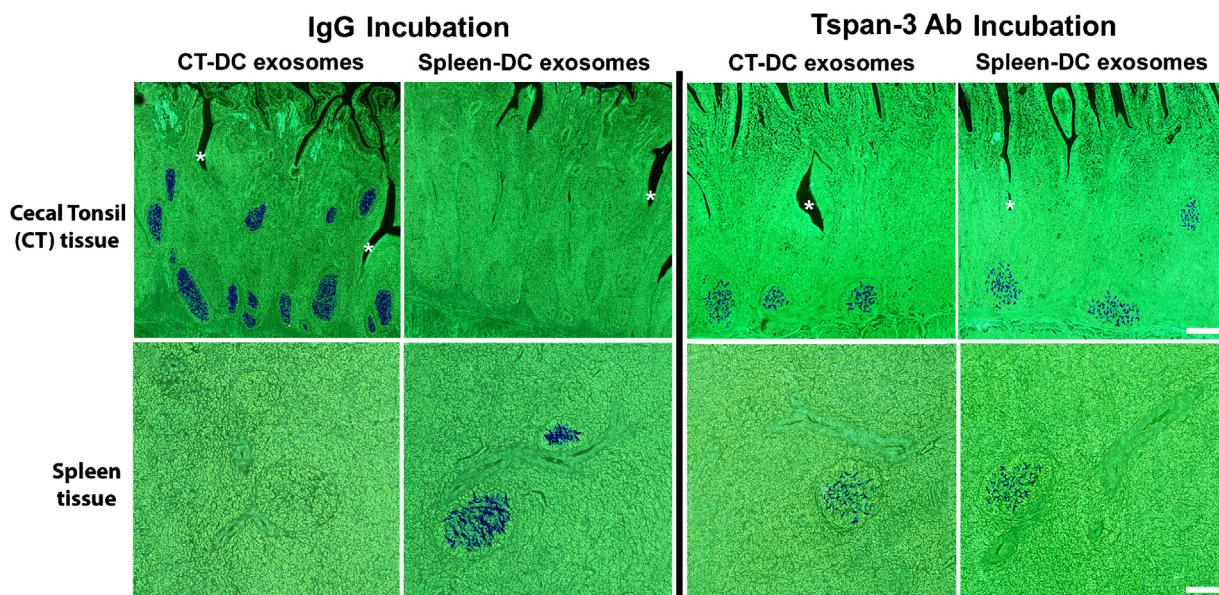


Fig. 2. Immunofluorescence lightmicrograph of *E. tenella* Ag in cecal tonsil or spleen sections from chickens immunized with Ag-loaded DC exosomes incubated with IgG or Tspan-3 Ab. DCs were isolated from cecal tonsils or spleen. Note the dense network of Ag in the germinal centres when exosomes were incubated with IgG compared with a loose network after exosome incubation with Tspan-3Ab. Asterisks (*) denote the lumen of the Lieberkühn crypts. Bars in cecal tonsil tissue = 150 μm. Bars in spleen tissue = 100 μm.

2.11. In vivo parameters of protection against *E. tenella* infection

Exosomes purified from tonsil and splenic DCs and incubated in the absence or presence of Tspan-3 Ab were injected

intramuscularly into chickens in groups 1–6 as described above. At 8 d post-immunization (15 d of age), the birds (group 5) were infected by oral gavage with a single dose of 1.0×10^4 *E. tenella* sporulated oocysts/bird. Chickens in group 6 served as

Table 1
Effects of immunization with *E. tenella* Ag-pulsed cecal tonsil or splenic DC exosomes incubated with Tspan-3 Ab or non-immune IgG on *in vivo* parameters of protection against *E. tenella* infection.

Experimental group	Body weight gain (g/chicken)	Feed conversion ratio (g feed/g chicken)	Fecal oocyst output ($\times 10^3$ /chicken)	Lesion score (0–4)	Mortality (%)
Unimmunized	662 ± 15^a	1.49 ± 0.10^a	0 ^a	0 ^a	0 ^a
Uninfected					
Unimmunized Infected	227 ± 35^b	4.32 ± 0.27^b	$15,028 \pm 0.57^b$	2.8 ± 0.7^b	5.5 ± 2.4^b
Cecal tonsil DC exosomes Incubation with IgG	642 ± 20^a	1.52 ± 0.13^a	785 ± 0.83^c	0.7 ± 0.5^c	0 ^a
Spleen DC exosomes Incubation with IgG	398 ± 34^c	2.87 ± 0.18^c	9573 ± 0.50^d	2.2 ± 0.7^b	0 ^a
Cecal tonsil DC exosomes Incubation with Tspan-3 Ab	527 ± 27^d	1.72 ± 0.10^d	2880 ± 0.48^e	1.5 ± 0.3^e	0 ^a
Spleen DC exosomes Incubation with Tspan-3 Ab	518 ± 31^d	1.75 ± 0.16^d	3322 ± 0.62^e	1.7 ± 0.5^e	0 ^a

Each value represents the mean \pm SD value of three independent experiments with each experiment containing 24 chickens/group. Within each group, values with different superscripts are significantly different ($P < 0.05$) according to the Duncan's multiple range test.

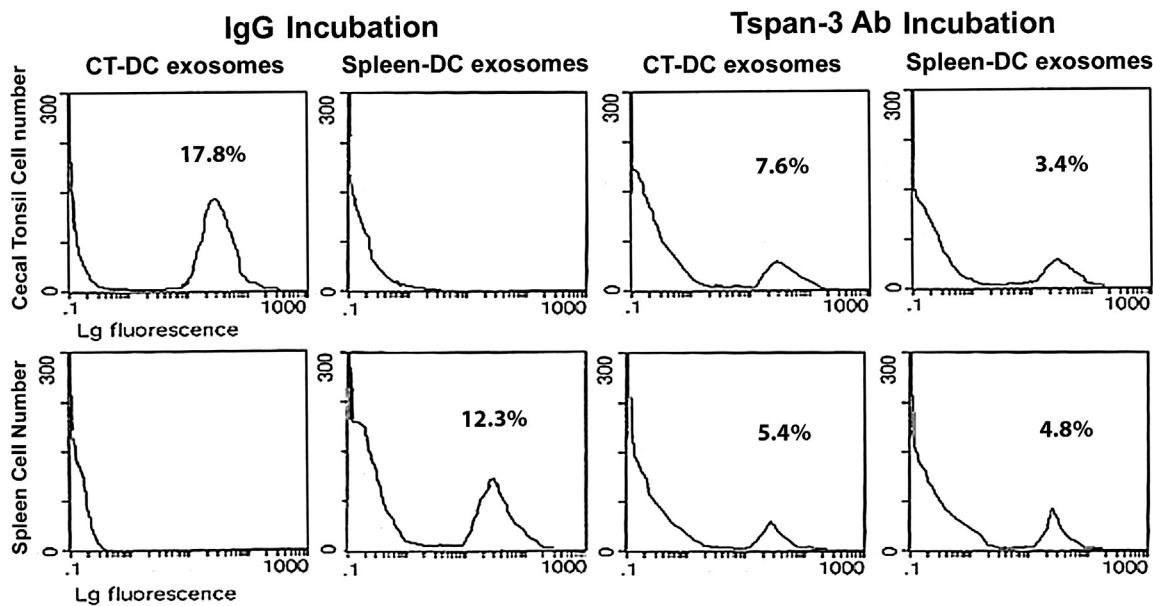


Fig. 3. *E. tenella* Ag FACS histograms of cells in cecal tonsils or spleen from chickens immunized with Ag-loaded DC exosomes incubated with IgG or Tspan-3 Ab. Ag loaded-DCs were isolated from the cecal tonsils or the spleen. The results are representative of three independent experiments.

unimmunized and uninfected controls. Chicken body weight gains and feed conversion ratios were measured between 0 and 10 d post-infection as described [25]. Fecal oocyst shedding was measured between 5 and 10 d post-infection as described [31]. At 6 d post-infection, lesion scores were determined by two independent observers as described [32]. The percent mortality in all groups was recorded at 10 d post-infection.

2.12. Statistical analysis

All values were expressed as means \pm SD. Differences between means were compared using the Student's *t*-test or ANOVA and were considered significant at $P < 0.05$. All experiments were repeated 3 times.

3. Results

3.1. Tspan-3 expression by DC exosomes

Immunogold staining of exosomes revealed the presence of gold particles on both cecal and splenic exosomes incubated with Tspan-3 Ab but not on those incubated with non-immune IgG (Fig. 1).

3.2. Immunofluorescence localization of *E. tenella* Ag within the cecal tonsils and spleen following administration of DC exosomes

The Ag-loaded exosomes were incubated with Tspan-3 Ab or IgG, injected intramuscularly into chickens, and cecal tonsils and spleens were examined by immunofluorescence microscopy after staining with *E. tenella* antiserum (Fig. 2). In animals injected with exosomes incubated with IgG, parasite Ag-containing cells were exclusively found in the cecal tonsils of chickens immunized with cecal DC exosomes (Fig. 2). Similarly, Ag-containing cells were only found in the spleens of chickens immunized with splenic DC exosomes (Fig. 2). By contrast, in chickens injected with exosomes incubated with Tspan-3 Ab, Ag-containing cells were observed in both the cecal tonsils and spleens of chickens immunized with exosomes from either cecal or splenic DCs (Fig. 2). The Ag-containing cells formed aggregates within the germinal centres (GCs). Interestingly, compared with exosomes incubated with IgG

fewer Ag-containing cells formed a loose network in the GCs of chickens immunized with exosomes with Tspan-3 Ab.

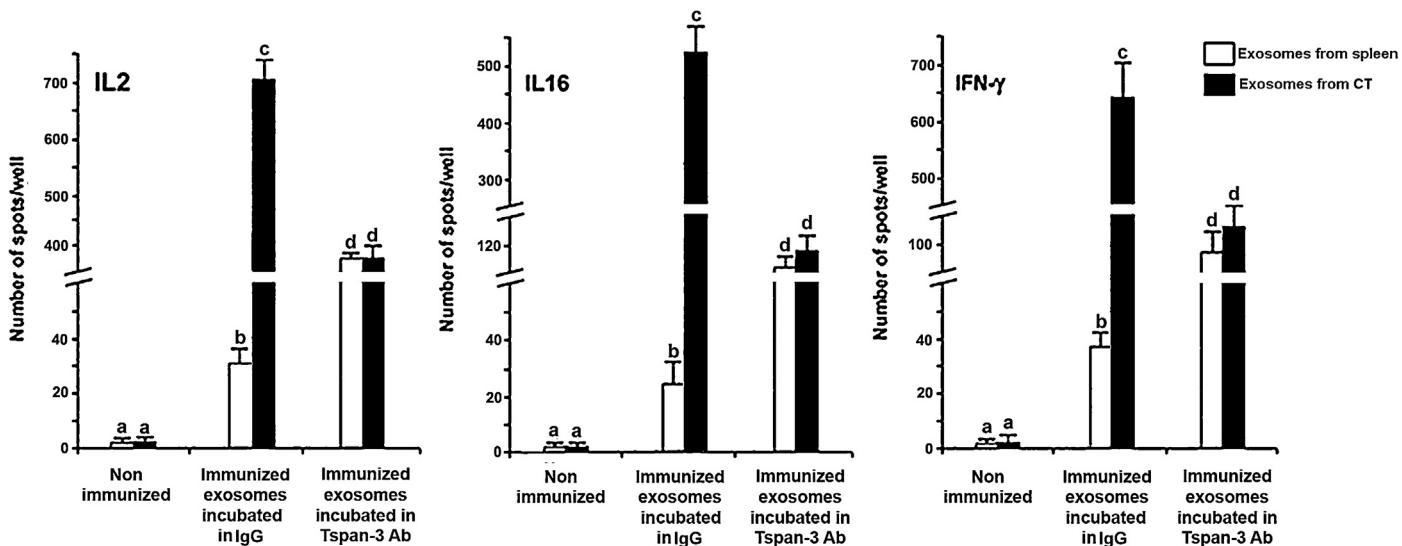
3.3. Quantification of *E. tenella* Ag containing cells in the cecal tonsils and spleen following administration of DC exosomes

The numbers of parasite Ag-containing cells in the cecal tonsils and spleen (Fig. 3) from chickens given exosomes derived from Ag-pulsed cecal or splenic DC were determined by flow cytometry. Immunostaining with *E. tenella* antiserum identified $17.8 \pm 4.2\%$ positive cells in the cecal tonsils of chickens injected with cecal DC exosomes incubated with IgG, but no immunostaining cells were detected in the spleens of chickens given cecal DC exosomes. Correspondingly, $12.3 \pm 3.8\%$ positive cells were seen in the spleen following administration of splenic DC exosomes incubated with IgG, while no Ag-containing cells were observed in the cecal tonsils of chickens given the splenic DC exosomes. As with the immunofluorescence microscopy results, after injection of exosomes incubated with Tspan-3 Ab, similar percentages of Ag-containing cells were found in the cecal tonsils and spleens, irrespective of the lymphoid organ from which the DCs were isolated.

3.4. Quantification of cytokine-producing cells

Cytokine secreting mononuclear cells were quantified by ELISPOT assay in cecal tonsils and spleens of chickens immunized with exosomes derived from Ag-pulsed cecal or splenic DC and incubated with Tspan-3 Ab or IgG (Fig. 4). When cecal tonsil exosomes incubated with IgG were injected, greater numbers of IL-2-, IL-16-, and IFN- γ -producing cecal tonsil cells were observed in chickens given the cecal tonsil DC exosomes compared with chickens immunized with splenic exosomes. By contrast, when splenic exosomes incubated with IgG were injected, more cytokine-producing spleen cells were seen compared with administration of cecal tonsil exosomes. After injection of exosomes incubated with Tspan-3 Ab, not only were the numbers of cytokine-producing cells in the cecal tonsils and spleens decreased compared with immunization with exosomes incubated with IgG, but also the numbers of

E. tenella Ag-specific Th1 response in Cecal Tonsil



E. tenella Ag-specific Th1 response in Spleen

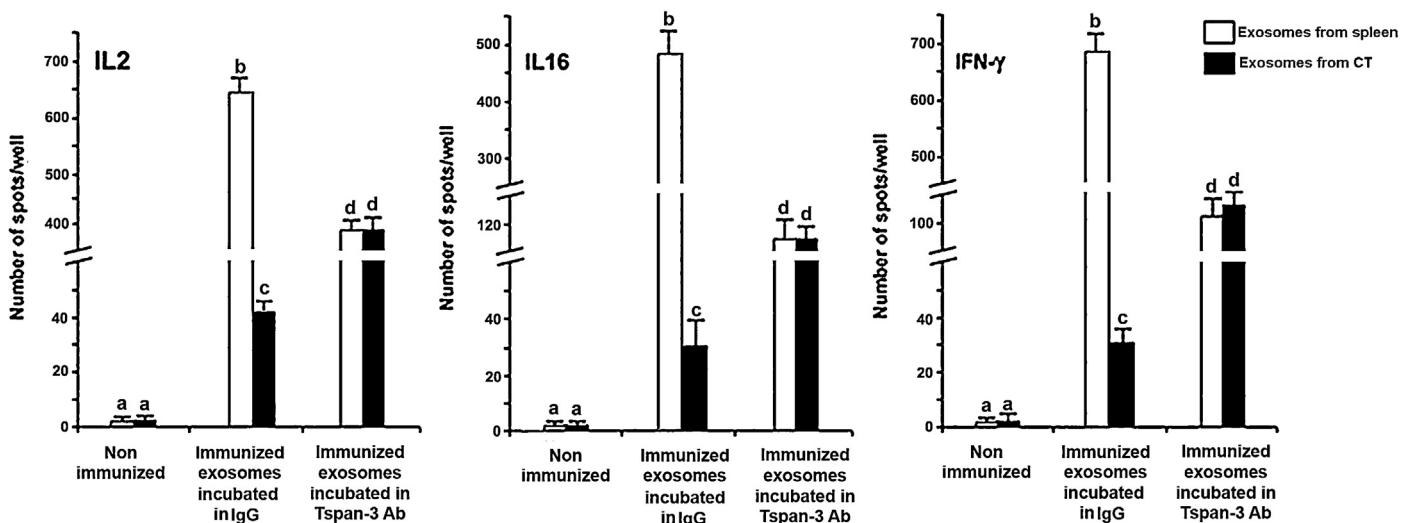


Fig. 4. *E. tenella* Ag-stimulated IL-2-, IL-16-, and IFN- γ -secreting cells in the cecal tonsils (upper panels) or spleens (lower panels) from chickens unimmunized or immunized with cecal (CT, closed bars) or spleen (open bars) exosomes which had been incubated with Tspan-3 Ab or IgG. Ag-stimulated cytokine-secreting cells were quantified by ELISPOT assay. Each bar represents the mean \pm SD value from three independent experiments. Within each graph, bars with different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.

cytokine-secreting cells in the cecal tonsils and spleen were similar regardless of whether the DCs were from the intestine or spleen.

3.5. Quantification of Ab-producing cells

E. tenella Ab-secreting cells were quantified by ELISPOT assay in cecal tonsils and spleen of chickens immunized with Ag-pulsed cecal or splenic DC exosomes, which had been incubated with Tspan-3 Ab or non-immune IgG (Fig. 5). In the absence of Tspan-3 Ab, greater numbers of *E. tenella* Ag-reactive IgG- and IgA-producing cecal tonsil cells were observed in chickens immunized with cecal tonsil DC exosomes compared with administration of splenic exosomes. Conversely, after injection of exosomes incubated with IgG, more IgG-producing spleen cells were seen in chickens immunized with splenic DC exosomes compared with injection of cecal tonsil exosomes. When exosomes incubated with

Tspan-3 Ab were injected, not only were the numbers of Ab-producing cells in the cecal tonsils and spleens lower compared with immunization with exosomes incubated with IgG, but also the numbers of Ab-secreting cells in the cecal tonsils and spleens were similar regardless of whether the DCs were from the cecal tonsil or spleen.

3.6. In vivo protection against *E. tenella* infection

Chickens were immunized with exosomes, which were derived from *E. tenella* Ag-pulsed DCs from cecal tonsils or spleen, and incubated with Tspan-3 Ab or IgG. Chickens were infected with *E. tenella* at 8 d post-immunization. Following parasite infection, body weight gain, feed conversion ratios, fecal oocyst shedding, and intestinal lesion scores were determined as parameters of protective immunity (Table 1). After immunization with exosomes

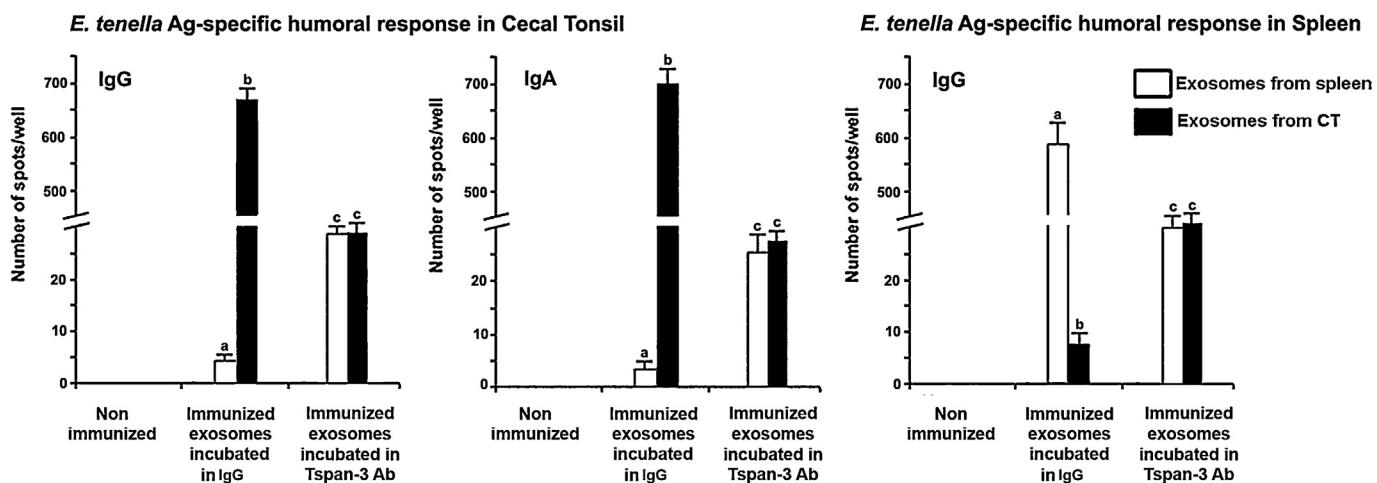


Fig. 5. *E. tenella* Ag-stimulated IgG- and IgA-secreting cells in the cecal tonsils (left two panels) or spleens (right panel) from chickens unimmunized or immunized with cecal (CT, closed bars) or spleen (open bars) exosomes which had been incubated with Tspan-3 Ab or IgG. Ag-reactive IgG- and IgA-secreting cells were quantified by ELISPOT assay. Each bar represents the mean \pm SD value from three independent experiments. Within each graph, bars with different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.

incubated with IgG, body weight gains were increased and feed conversion ratios, fecal oocyst shedding, and intestinal lesion scores were decreased in infected chickens immunized with cecal tonsil DC exosomes compared with unimmunized and infected controls, indicating the generation of a protective response by the exosomes. Similar effects were seen in infected chickens immunized with splenic DC exosomes compared with unimmunized and infected controls, although the magnitudes of these parameters were not as great as following immunization with cecal DC exosomes. When exosomes incubated with Tspan-3 Ab were injected, body weight gains were decreased and feed conversion ratios, oocyst shedding, and gut lesion scores were increased in infected chickens immunized with cecal tonsil DC exosomes compared with immunization by injection of exosomes incubated with IgG. These results suggested that the Tspan-3 Ab blocked the host protective response to *E. tenella* infection induced by the cecal DC exosomes. However, all four parameters remained significantly different in immunized and infected chickens which were immunized with exosomes incubated with Tspan-3 Ab compared with unimmunized and uninfected controls, indicating that the Tspan-3Ab only partially blocked protection against parasite infection. Interestingly, weight gains were increased and conversion ratios, oocyst shedding, and lesion scores were decreased in infected chickens immunized with splenic DC exosomes incubated with Tspan-3 Ab compared with immunization with exosomes incubated with IgG, indicative of a greater protective response to infection.

4. Discussion

Avian coccidiosis is a major infectious disease with high economic impact on the commercial poultry industry [33]. In the absence of treatment, ingestion of infectious *Eimeria* parasites leads to a necrotizing intestinal tissue destruction characterized by reduced feed conversion efficiency, decreased body weight gain, and a vicious cycle of parasite intake, gut infection, fecal excretion, and re-infection. Attenuated parasite vaccines constitute one avenue of disease control, and Ag-loaded DC exosomes, in particular, offers a novel, related immunization strategy [26,34]. However, the molecular and cellular mechanisms through which exosomes confer resistance to avian coccidiosis are poorly understood. In this report, we now have established that Tspan-3 is a regulator of exosomal vaccination efficacy in the experimental model of *E. tenella* avian coccidiosis.

Incubation of exosomes with Tspan-3 Ab blocked their tissue-specific localization to the lymphoid organ from which they were derived, decreased the numbers of cytokine- and Ab-producing cells in these target organs, and improved multiple *in vivo* parameters of disease resistance. These results suggest that this membrane-spanning protein controls host immunity to experimental avian coccidiosis. These findings are consistent with the results of Beauvillain et al. [13] who demonstrated a preferential homing of splenic DC exosomes to the spleen. Other studies have shown that DC exosomes are efficiently taken up only by DC lineage cells [35]. Exosomes from different cellular sources bear proteins specific for their cell type of origin and these proteins, as well as those of their target cells, mediate exosomal uptake [3,36]. The results of the present study in an avian model system support a mechanistic basis for target cell selection by exosomes invoking Tspan proteins as entry selection markers.

While prior studies have demonstrated that Tspans are present on mammalian DC exosomes, this report is the first to document Tspan-3 expression by DC exosomes and that Tspan-3 regulates the post-injection tissue distribution of exosomes, as well as *in vitro* and *in vivo* parameters of exosome-dependent immunity to an intracellular parasite.

Previous investigations have yielded important new insights into the nature of the immune response induced by Ag-loaded exosomes against intracellular pathogens [37]. DC exosomes mediate Ag presentation to T cells, resulting in Th1 Ag-specific immune responses [13,21,38,39]. The present findings reveal that the efficiency of immunity against *Eimeria* parasites is dependent upon the tissue site from which the DC exosomes were prepared. While administration of splenic DC exosomes induced weak protection against subsequent *E. tenella* infection, exosomes released from cecal DCs stimulated a strong intestinal immune response against the parasite. Given that *E. tenella* colonizes the ceca of chickens, a plausible explanation for these results is that in order for exosomes to stimulate an effective immune response against the invading parasites, they must be released by donor DCs homing to the specific tissue responsible for host protective immunity. This hypothesis is of obvious relevance for the use of Ag-loaded exosomes as vaccines designed for the preferential stimulation of local vs. systemic immunity.

Exosome vaccines offer several advantages over conventional vaccination regimens. Exosomes can be produced in large quantities under good manufacturing practices and remain stable for

extended times when cryopreserved [9]. Further, single exosome preparations can be loaded with diverse Ags from a variety of pathogens to produce multivalent vaccines. It was originally anticipated that Ag-containing exosome vaccines would require immunization of individuals syngeneic at the MHC with the donor cells [40,41]. However, MHC barriers do not restrict exosome efficacy since syngeneic and allogeneic exosomes are equally effective as vaccines [8,13,26,34]. Therefore, although the physiopathological relevance of DC exosomes is unclear, their potential for vaccinology is great. In conclusion, the results of the current study suggest that it may be possible to produce an efficacious, exosome-based coccidiosis vaccine for future use by commercial poultry producers. In addition, the current results indicate that isolation of exosomes from DCs within the lymphoid organ which plays the main role in the response against the parasite infection may provide a superior vaccine formulation.

Acknowledgments

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